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(71) Applicant: MICHIGAN STATE UNIVERSITY [US/US]; 238 Administration Building, East Lansing, MI 48824 (US).

(72) Inventors: THOMASHOW, Michael, F.; 805 Southlawn, East Lansing, MI 48823 (US). STOCKINGER, Eric, J.; 1360 Burcham Drive, East Lansing, MI 48823 (US).

(74) Agent: McLEOD, Ian, C.; 2190 Commons Parkway, Okemos, MI 48864 (US).

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(54) Title: DNA AND ENCODED PROTEIN WHICH REGULATES COLD AND DEHYDRATION REGULATED GENES

(57) Abstract

A gene, designated as *CBF1*, encoding a protein, *CBF1*, which binds to a region regulating expression of genes which promote cold temperature and dehydration tolerance in plants is described. *CBF1* is used to transform microorganisms and can be used to transform plants.

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DNA AND ENCODED PROTEIN WHICH REGULATES COLD AND DEHYDRATION REGULATED GENES

BACKGROUND OF THE INVENTION

(1) Summary of the Invention

The present invention relates to a gene, *CBF1*, encoding a protein, designated as CBF1, which binds to a region regulating expression of genes which are activated during acclimation to cold temperature and drought. The gene is expressed in microorganisms and can be used to produce recombinant plants.

(2) Description of Related Art

Environmental factors serve as cues to trigger a number of specific changes in plant growth and development. One such factor is low temperature. Prominent examples of cold-regulated processes include cold acclimation, the increase in freezing tolerance that occurs in response to low non-freezing temperatures (Guy, C. L., Annu. Rev. Plant Physiol. Plant Mol. Biol. **41**:187-223 (1990)); vernalization, the shortening of time to flowering induced by low temperature (Lang, A., in Encyclopedia of Plant Physiology, Vol. 15-1, ed. Ruhland, W. (Springer, Berlin), pp. 1489-1536 (1965)); and stratification, the breaking of seed dormancy by low temperature (Berry, J. A. and J. K. Raison, in Encyclopedia of Plant Physiology, Vol. 12A, eds. Lange, O. L., Nobel, P. S., Osmond, C. B. and Ziegler, H. (Springer, Berlin), pp. 277-338 (1981)). Due to the fundamental nature and agronomic importance of these processes, there is interest in understanding how plants sense and respond to low temperature. One approach being taken is to determine the signal transduction pathways and regulatory mechanisms involved in cold-regulated gene expression.

Strong evidence exists for calcium having a role in low temperature signal transduction and regulation of at least some COR (cold-regulated) genes. Dhindsa and colleagues (Monroy, A. F., et al, Plant Physiol. **102**:1227-1235 (1993); Monroy, A. F., and R. S., The Plant Cell, **7**:321-331 (1995)) have shown that, in alfalfa, calcium chelators and calcium channel blockers prevent low temperature induction of COR genes and that calcium ionophores and calcium channel agonists induce expression of COR genes at normal growth temperatures. Similarly, Knight et al (The Plant Cell **8**:489-503 (1996)) have shown that cold-induced expression of the *Arabidopsis thaliana* COR gene *KIN1* is inhibited by calcium chelators and calcium channel blockers. These results suggest that low temperature triggers an influx of extracellular calcium that activates a signal transduction pathway that induces the expression of COR genes. Consistent with this notion is the finding that low temperature evokes transient increases in cytosolic calcium levels in plants (Knight, M. R. et al, Nature **352**:524-526 (1991); Knight, H., et al., The Plant Cell **8**:489-503 (1996)). In addition, low temperatures have been shown to stimulate the activity of mechanosensitive calcium-selective cation channels in plants (Ding, J. P. and B. G. Pickard, Plant J. **3**:713-720 (1993)).

Recent efforts have led to the identification of a *cis*-acting cold-regulatory element in plants, the C-repeat/DRE (Yamaguchi-Shinozaki, et al., The Plant Cell **6**:251-264 (1994); Baker, S. S., et al., Plant. Mol. Biol. **24**:701-713 (1994); Jiang, C., et al., Plant Mol. Biol. **30**:679-684 (1996)). The element, which has a 5 base pair core sequence for CCGAC, is present once to multiple times in all plant cold-regulated promoters that have been described to date; these include the promoters of the *COR15a* (Baker, S. S., et al, Plant. Mol. Biol. **24**:701-713 (1994)), *COR78/RD29A* (Horvath, D. P., et al, Plant Physiol. **103**:1047-1053 (1993);

Yamaguchi-Shinozaki, K., et al., The Plant Cell **6**:251-264 (1994)), *COR6.6* (Wang, H., et al., Plant Mol. biol. **28**:605-617 (1995)) and *KIN1* (Wang, H., et al, Plant Mol. Biol. **28**:605-617 (1995)) genes of Arabidopsis and the *BN115* gene of *Brassica napus* (White, T. C., et al, Plant Physiol. **106**:917-928 (1994)). Deletion analysis of the Arabidopsis *COR15a* gene suggested that the CCGAC sequence, designated the C-repeat, might be part of a *cis*-acting cold-regulatory element (Baker, S. S., et al., Plant Mol. Biol. **24**:701-713 (1994)). That this was the case was first demonstrated by Yamaguchi-Shinozaki and Shinozaki (Yamaguchi-Shinozaki, K., et al., The Plant Cell **6**:251-264 (1994)) who showed that two of the C-repeat sequences present in the promoter of *COR78/RD29A* induced cold-regulated gene expression when fused to a reporter gene. It was also found that these two elements stimulate transcription in response to dehydration and high salinity and thus, was designated the DRE (dehydration, low temperature and high salt regulatory element). Interestingly, two other C-repeats present in the promoter of *COR78/RD29A* did not impart regulation in response to dehydration and high salt; whether they impart cold-regulated gene expression is not known. Recent studies by Jiang et al (Jiang, C., et al., Plant Mol. Biol. **30**:679-684 (1996)) indicate that the C-repeats (referred to as low temperature response elements) present in the promoter of the *B. napus BN115* gene also impart cold-regulated gene expression, but do not activate gene expression in response to dehydration.

U.S. Patents Nos. 5,296,462 and 5,356,816 to Thomashow describe the genes encoding the proteins involved in cold regulation in *Arabidopsis thaliana*. In particular the DNA encoding the COR15 proteins is described. These proteins are significant in promoting cold tolerance in plants; however, the mode of activation is not described.

There is a need for a protein which regulates

the expression of genes encoding cold and drought tolerance genes.

OBJECTS

Therefore, it is an object of the present invention to provide a protein and gene encoding the protein which regulates expression of genes activated during acclimation to cold temperature and dehydration stress. It is further an object of the present invention to provide a unique protein and DNA sequences encoding the protein. These and other objects will become increasingly apparent by reference to the following description and the drawings.

IN THE DRAWINGS

Figures 1A and 1B show how the yeast reporter strains were constructed. Figure 1A is a schematic diagram showing the screening strategy. Yeast reporter strains were constructed that carried C-repeat/DRE sequences as UAS elements fused upstream of a *lacZ* reporter gene with a minimal *GAL1* promoter. The strains were transformed with an Arabidopsis expression library that contained random cDNA inserts fused to the GAL4 activation domain (GAL4-ACT) and screened for blue colony formation on X-gal-treated filters. Figure 1B is a chart showing activity of the "positive" cDNA clones in yeast reporter strains. The oligonucleotides (oligos) used to make the UAS elements, and their number and direction of insertion, are indicated by the arrows.

Figures 2A, 2B, 2C and 2D provide an analysis of the pACT-11 cDNA clone. Figure 2A is a schematic drawing of the pACT-11 cDNA insert indicating the location and 5' to 3' orientation of the 24 kDa polypeptide and 25s rRNA sequences. The cDNA insert was cloned into the *Xho*I site of the pACT vector. Figure 2B is a DNA and amino acid sequence of the 24 kDa polypeptide (SEQ ID NO:1 and SEQ ID NO:2). The AP2 domain is indicated by a double underline. The basic amino acids that potentially act as a nuclear

localization signal are indicated with asterisks. The *Bcl*I site immediately upstream of the 24 kDa polypeptide used in subcloning the 24 kDa polypeptide and the *Eco*RV site used in subcloning the 3' end of CBF1 are indicated by single underlines. Figure 2C is a schematic drawing indicating the relative positions of the potential nuclear localization signal (NLS), the AP2 domain and the acidic region of the 24 kDa polypeptide. Numbers indicate amino acid residues. Figure 2D is a chart showing comparison of the AP2 domain of the 24 kDa polypeptide with that of the tobacco DNA binding protein EREBP2 (Okme-Takagi, M., et al., The Plant Cell 7:173-182 (1995) SEQ ID NOS: 10 and 11). Identical amino acids are indicated with single lines; similar amino acids are indicated by double dots; amino acids that are invariant in AP2 domains are indicated with asterisks (Klucher, K. M., et al., The Plant Cell 8:137-153 (1996)); and the histidine residues present in CBF1 and TINY (Wilson, K., et al., The Plant Cell 8:659-671 (1996)) that are tyrosine residues in all other described AP2 domains are indicated with a caret. A single amino acid gap in the CBF1 sequence is indicated by a single dot.

Figure 3 is a chart showing activation of reporter genes by the 24 kDa polypeptide. Restriction fragments of pACT-11 carrying the 24 kDa polypeptide (*Bcl*I-*Bgl*II) or the 24 kDa polypeptide plus a small amount of 25s RNA sequence (*Bgl*II-*Bgl*II) were inserted in both orientations into the yeast expression vector pDB20.1 (see Figure 2A and 2B for location of *Bcl*I and *Bgl*II restriction sites). These "expression constructs" were transformed into yeast strains carrying the *lacZ* reporter gene fused to direct repeat dimers of either the wild-type *COR15a* C-repeat/DRE (oligonucleotide MT50) or the mutant M2*COR15a* C-repeat/DRE (oligonucleotide MT80). The specific activity of β-galactosidase (nmoles o-nitrophenol produced/min^{-1} x mg protein $^{-1}$) was

determined from cultures grown in triplicate. Standard deviations are indicated. Abbreviations: pADC1, *ADC1* promoter; tADC1, *ADC1* terminator.

Figure 4 is a photograph of an electrophoresis gel showing expression of the recombinant 24 kDa polypeptide in *E. coli*. Shown are the results of SDS-PAGE analysis of protein extracts prepared from *E. coli* harboring either the expression vector alone (vector) or the vector plus an insert encoding the 24 kDa polypeptide in sense (sense insert) or antisense (antisense insert) orientation. The 28 kDa fusion protein (see Materials and Methods) is indicated by an arrow.

Figure 5 is a photograph of a gel for shift assays indicating that CBF1 binds to the C-repeat/DRE. The C-repeat/DRE probe (1 ng) used in all reactions was a ^{32}P-labeled dimer of the oligonucleotide MT50 (wild type C-repeat/DRE from *COR15a*). The protein extracts used in the first four lanes were either bovine serum albumin (BSA) or the indicated CBF1 sense, antisense and vector extracts described in Figure 4. The eight lanes on the right side of the figure used the CBF1 sense protein extract plus the indicated competitor C-repeat/DRE sequences (100 ng). The numbers 1X, 2X and 3X indicate whether the oligonucleotides were monomers, dimers or trimers, respectively, of the indicated C-repeat/DRE sequences.

Figure 6 is a photograph of a southern blot analysis indicating *CBF1* is a unique or low copy number gene. Arabidopsis DNA (~1μg) was digested with the indicated restriction endonucleases and southern transfers were prepared and hybridized with a ^{32}P-labeled probe encoding the entire CBF1 polypeptide.

Figures 7A, 7B and 7C relate to *CBF1* transcripts in control and cold-treated Arabidopsis. Figure 7A is a photograph of a membrane RNA isolated from Arabidopsis plants that were grown at 22°C or grown

at 22°C and transferred to 2.5°C for the indicated times. Northern transfers were prepared from either total RNA (10 μg) or poly(A)$^{+}$ RNA (1 μg) and hybridized with ^{32}P-labeled probes for the entire *CBF1* coding sequence or the cold-regulated *COR15a* gene, respectively. The membranes were stripped and re-probed with ^{32}P-labeled pHH25, a cDNA encoding the small subunit of rubisco. The transcript levels for pHH25 are about the same in control and cold-treated plants (Hajela, R. K., et al., Plant Physiol. **93**:1246-1252 (1990)). Figures 7B and 7C are graphs showing relative transcript levels of *CBF1* and *COR15a* in control and cold-treated plants. The radioactivity present in the samples described in Figure 7A were quantified using a Betascope 603 blot analyzer and plotted as relative transcript levels (the values for the 22°C grown plants being arbitrarily set as 1) after adjusting for differences in loading using the values obtained with the pHH25 probe.

<u>Description of Preferred Embodiments</u>.

The present invention relates to an isolated DNA encoding a protein having a sequence of amino acids essentially homologous to that set forth in SEQ ID NO:2 such that upon providing the DNA encoding the protein in a living plant material a transcription regulating region controlling at least one cold or dehydration regulated gene in the plant material is activated.

The present invention also relates to a plasmid deposited as ATCC 98063 containing a DNA insert encoding a protein which activates a transcription regulating region identified as the C-repeat/DRE.

The present invention also relates to a plasmid containing a DNA insert encoding a protein comprising a sequence of amino acids essentially homologous to that set forth in SEQ ID NO:2 such that upon providing the DNA encoding protein in a living plant material a transcription regulating region controlling at least one cold or dehydration regulated

gene in the plant material is activated.

The present invention also relates to a microorganism containing a plasmid having a DNA insert encoding a protein comprising a sequence of amino acids essentially homologous to that set forth in SEQ ID NO:1 such that upon providing the DNA in a living plant material a transcription regulating region controlling at least one cold or dehydration regulated gene in the living plant material is activated.

The present invention also relates to a plant material containing a recombinant DNA encoding a protein comprising a sequence of amino acids essentially homologous to that set forth in SEQ ID NO:1 such that a transcription regulating region controlling at least one cold or dehydration regulated gene in the plant material is activated by the protein.

The present invention also relates to a protein free of other proteins comprising a sequence of amino acids essentially homologous to that set forth in SEQ ID NO:2 such that upon providing DNA encoding the protein in a living plant material a transcription regulating region controlling at least one cold or dehydration regulated gene in the plant material is activated.

The present invention also relates to a protein free of other proteins having an amino acid sequence as set forth in SEQ ID NO:2.

The present invention also relates to a protein free of other proteins of *Arabidopsis thaliana* having a molecular weight of about 24 kD as measured in an electrophoresis gel, which binds to a transcription regulating DNA region controlling a cold or dehydration regulated gene of the *Arabidopsis thaliana*.

The present invention also relates to a protein as encoded by a DNA in plasmid pEJS251 deposited as ATCC 98063.

The present invention also relates to a

composition useful for introduction into plants to increase cold or dehydration tolerance of the plant which comprises:

(a) a protein having a sequence of amino acids essentially homologous to that set forth in SEQ ID NO:2 such that upon providing the DNA encoding the protein in a living plant material a transcription regulating region controlling at least one cold or dehydration regulated gene in the plant material is activated; and

(b) a carrier for the introduction of the protein into the plant.

The present invention relates to a composition useful for introduction into plants to increase cold or dehydration tolerance of the plant which comprises:

(a) a protein as encoded by a DNA in a plasmid deposited as ATCC 98063; and

(b) a carrier for the introduction of the protein into the plant.

The present invention relates to a method of regulating cold or dehydration tolerance of a living plant material which comprises:

introducing into the living plant material a DNA encoding a protein comprising a sequence of amino acids essentially homologous to that set forth in SEQ ID NO:1 such that upon providing the DNA encoding the protein in the living plant material a transcription regulating region controlling at least one cold or dehydration regulated gene in the plant material is activated.

The term "cold" includes freezing temperatures as well as more elevated temperatures less than ambient temperatures. The term "dehydration" includes salt stress and osmotic stress.

Transformation means the process for changing the genotype of a recipient organism by the stable introduction of DNA by whatever means.

A transgenic plant is a plant which contains DNA sequences which were introduced by transformation. Horticultural and crop plants particularly benefit from the present invention.

Translation means the process whereby the genetic information in an mRNA molecule directs the order of specific amino acids during protein synthesis.

The term "essentially homologous" means that the DNA or protein is sufficiently duplicative of that set forth in Figure 2B to produce the same result. Such DNA can be used as a probe to isolate DNA's in other plants.

A promoter is a DNA fragment which causes transcription of genetic material. For the purposes described herein, promoter is used to denote DNA fragments that permit transcription in plant cells.

A poly-A addition site is a nucleotide sequence which causes certain enzymes to cleave mRNA at a specific site and to add a sequence of adenylic acid residues to the 3'-end of the mRNA.

In the following description, the isolation of an *Arabidopsis thaliana* cDNA clone that encodes a C-repeat/DRE binding factor, CBF1 (C-repeat/DRE Binding Factor 1) is described. Expression of CBF1 in yeast activated transcription of reporter genes containing the C-repeat/DRE as an upstream activator sequence, but not mutant versions of the element, indicating that CBF1 is a transcription factor that binds to the C-repeat/DRE. Binding of CBF1 to the C-repeat/DRE was also demonstrated in gel shift assays using recombinant CBF1 protein expressed in *Escherichia coli*. Transcript levels of *CBF1* - which is a single or low copy number gene - increased only slightly (2 to 3 fold) in response to low temperature. Analysis of the deduced CBF1 amino acid sequence indicated that the protein has a potential nuclear localization sequence, a possible acidic activation domain and an AP2 domain, a DNA-binding motif

of about 60 amino acids that is similar to those present in Arabidopsis proteins APETALA2, AINTEGUMENTA and TINY, the tobacco ethylene response element binding proteins, and numerous other plant proteins of unknown function. Implications of CBF1 activating transcription in yeast and possible mechanisms of regulating CBF1 activity in Arabidopsis are discussed.

Escherichia coli strain GM2163 containing plasmid pEJS251 was deposited under the Budapest Treaty on May 17, 1996 with the American Type Culture Collection, Rockville, Maryland as ATCC 98063. It is available by name and number pursuant to the provisions of the Budapest Treaty.

EXAMPLE 1

Materials and Methods

Plant material and cold treatment. *A thaliana* (L.) Heyn. ecotype RLD plants were grown in pots in controlled environment chambers at 22°C under constant illumination with cool-white fluorescent lamps (~100 μmol $m^{-2}s^{-1}$) essentially as described (Gilmour, S. J., Plant Physiol. **87**:745-750 (1988)). Plants were cold-treated by placing pots in a cold room at 2.5°C under constant illumination with cool-white florescent lamps (~25 μmol $m^{-2}s^{-1}$) for the indicated times.

Yeast reporter strains. Oligonucleotides (Table 1) (synthesized at the MSU Macromolecular Structure Facility) encoding either wild-type or mutant versions of the C-repeat/DRE were ligated into the *Bgl*II site of the *lacZ* reporter vector pBgl-lacZ (Li, J. J. and I. Herskowitz, Science **262**:1870-1874 (1993); kindly provided by Joachim Li).

TABLE 1

Oligonucleotides encoding wild type and mutant versions of the C-repeat/DRE

Oligonucleotide	C-repeat /DRE*	Sequence	SEQ ID NO:
MT50	*COR15a*	gatcATTTCATGG**CCGAC**CTGCTTTTT	3
MT52	M1*COR15a*	CACAATTTCAaGaattcaCTGCTTTTTT	4
MT80	M2*COR15a*	gatcATTTCATGGtatgtCTGCTTTTT	5
MT125	M3*COR15a*	gatcATTTCATGGaatcaCTGCTTTTT	6
MT68	*COR15b*	gatcACTTGATGG**CCGAC**CTCTTTTTT	7
MT66	*COR78*-1	gatcAATATACTA**CCGAC**ATGAGTTCT	8
MT86	*COR78*-2	ACTA**CCGAC**ATGAGTTCCAAAAAGC	9

*The C-repeat/DRE sequences tested are either wild-type found in the promoters of *COR15a* (Baker, S. S., et al., Plant. mol. Biol. **24**:701-713 (1994)), *COR15b* or *COR78/RD29A* (Horvath, D. P., et al., Plant Physiol. **103**:1047-1053 (1993); Yamaguchi-shinozaki, K., et al., The Plant Cell **6**:251-264 (1994)) or are mutant versions of the *COR15a* C-repeat/DRE (M1*COR15a*, M2*COR15a* and M3*COR15a*).

#Uppercase letters designate bases in wild type C-repeat/DRE sequences. The core CCGAC sequence common to all C-repeats is indicated in bold type. Lowercase letters at the beginning of a sequence indicate bases added to facilitate cloning. The lowercase letters that are underlined indicate the mutations in the C-repeat/DRE sequence of *COR15a*.

The resulting reported constructs were integrated into the *ura3* locus of *Saccharomyces cerevisiae* strain GGY1 (*MATα Δgal4 Δgal80 ura3 leu2 his3 ade2 tyr*) (Li, J. J. and I. Herskowitz, Science **262**:1870-1874 (1993); provided by Joachim Li) by transformation and selection for uracil prototrophy.

Screen of Arabidopsis cDNA library. The Arabidopsis pACT cDNA expression library that was constructed by John Walker and colleagues (NSF/DOE/USDA Collaborative Research in Plant Biology Program grant USDA 92-37105-7675) and deposited in the Arabidopsis Biological Resource Center (stock #CD4-10) was screened for clones encoding C-repeat/DRE binding domains. The cDNA library, harbored in *Escherichia coli* BNN132, was amplified by inoculating 0.5 ml of the provided glycerol stock into 1 L of M9 minimal glucose medium (Sambrook, J. et al, Molecular Cloning. A Laboratory Manual (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY., 2nd Ed. (1989)) and shaking the bacteria for 20 h at 37°C. Plasmid DNA was isolated and purified by cesium chloride density gradient centrifugation (Sambrook et al (1989)) and transformed into the yeast GGY1 reporter strains selecting for leucine prototrophy. Yeast transformants that had been grown for 2 or 3 days at 30°C were overlaid with either a nitrocellulose membrane filter (Schleicher and Schuell, Keene, NH) or Whatman #50 filter paper (Hillsboro, OR) and incubated overnight at 30°C. The yeast impregnated filters were then lifted from the plate and treated with X-gal (5-bromo-4-chloro-3-indolyl β-D-galactosidase) to assay colonies for β-galactosidase activity (Li, J. J. and I. Herskowitz, Science **262**:1870-1874 (1993)). Plasmid DNA from "positive" transformants (those forming blue colonies on the X-gal-treated filters) was recovered (Strathern, J. N., and D. R. Higgens, Methods Enzymol. **194**:319-329 (1991)), propagated in *E. coli* DH5α and transformed back into the yeast reporter strains to confirm activity.

Yeast transformation and quantitative β-galactosidase assays. Yeast were transformed by either electroporation (Becker, D. M., et al., Methods Enzymol. **194**:182-187 (1991)) or the lithium acetate/carrier DNA method (Schiestl, R. H., et al., Current Genetics **16**:339-346 (1989)). Quantitative *in vitro* β-galactosidase assays were done as described (Rose, M., et al., Methods Enzymol. **101**:167-180 (1983)).

Expression of CBF1 protein in *E. coli* and yeast. CBF1 was expressed in *E. coli* using the pET-28a(+) vector (Novagen, Madison, WI). The *Bgl*II-*Bcl*I restriction fragment of pACT-11 encoding CBF1 was ligated into the *Bam*HI site of the vector bringing CBF1 under control of the T7 phage promoter. The construct resulted in a "histidine tag," a thrombin recognition sequence and a "T7 epitope tag" being fused to the amino terminus of CBF1. The construct was transformed into *E. coli* BL21 (DE3) and the recombinant CBF1 protein was expressed as recommended by the supplier (Novagen). Expression of CBF1 in yeast was accomplished by ligating restriction fragments encoding CBF1 (the *Bcl*I-*Bgl*II and *Bgl*II-*Bgl*II fragments from pACT-11) into the *Bgl*II site of pDB20.1 (Berger, S. L., et al., Cell **70**:251-265 (1992); kindly provided by Steve Triezenberg) bringing CBF1 under control of the constitutive *ADC1* (alcohol dehydrogenase constitutive 1) promoter.

Gel shift assays. Total soluble *E. coli* protein (40 ng) was incubated at room temperature in 10 μl of 1X binding buffer [15 mM HEPES (pH 7.9), 1 mM EDTA, 30mM KCl, 5% glycerol, 5% BSA, 1mM DTT) plus 50 ng poly(dI-dC):poly(dI-dC) (Pharmacia, Piscataway, NJ) with or without 100 ng competitor DNA. After 10 min, probe DNA (1 ng) that was ^{32}P-labeled by end-filling (Sambrook et al, 1989) was added and the mixture incubated for an additional 10 min. Samples were loaded onto polyacrylamide gels (4% w/v) and fractionated by electrophoresis at 150V for 2h (Sambrook et al). Probes

and competitor DNAs were prepared from oligonucleotide inserts ligated into the *Bam*HI site of pUC118 (Vieira, J., et al., Methods Enzymol. **153**:3-11 (1987)). Orientation and concatenation number of the inserts were determined by dideoxy DNA sequence analysis (Sambrook, et al, (1989)). Inserts were recovered after restriction digestion with *Eco*RI and *Hind*III and fractionation on polyacrylamide gels (12% w/v) (Sambrook et al, 1989).

Northern and southern analysis. Total RNA was isolated from Arabidopsis (Gilmour, S. J., et al., Plant Physiol. **87**:745-750 (1988)) and the poly(A)$^+$ fraction purified using oligo dT cellulose (Sambrook, et al (1989)). Northern transfers were prepared and hybridized as described (Hajela, R. K., et al., Plant physiol. **93**:1246-1252 (1990)) except that high stringency wash conditions were at 50°C in 0.1X SSPE [X SSPE is 3.6 M NaCl, 20 mM EDTA, 0.2 M Na_2-HPO_4 (pH7.7)], 0.5% SDS. Membranes were stripped in 0.1 X SSPE, 0.5% SDS at 95°C for 15 min prior to re-probing. Total Arabidopsis genomic DNA was isolated (Stockinger, E. J., et al., J. Heredity, **87**:214-218 (1996)) and southern transfers prepared (Sambrook et al 1989) using nylon membranes (MSI, Westborough, MA). High stringency hybridization and wash conditions were as described by Walling et al (Walling, L. L., et al., Nucleic Acids Res. **16**:10477-10492 (1988)). Low stringency hybridization was in 6X SSPE, 0.5% SDS, 0.25% low fat dried milk at 60°C. Low stringency washes were in 1X SSPE, 0.5% SDS at 50°C. Probes used for the entire CBF1 coding sequence and 3' end of CBF1 were the *Bcl*I/*Bgl*II and *Eco*RV/*Bgl*II restriction fragments from pACT-11, respectively, that had been gel purified (Sambrook et al (1989)). DNA probes were radiolabeled with ^{32}P-nucleotides by random priming (Sambrook). Autoradiography was performed using hyperfilm-MP (Amersham, Arlington Heights, IL). Radioactivity was

quantified using a Betascope 603 blot analyzer (Betagen Corp., Waltham, MA).

RESULTS

Screen for Arabidopsis cDNAs encoding a C-repeat/DRE binding domain. The "one-hybrid" strategy (Li, J. J. and I. Herskowitz, Science **262**:1870-1874 (1993)) was used to screen for Arabidopsis cDNA clones encoding a C-repeat/DRE binding domain. In brief, yeast strains were constructed that contained a *lacZ* reporter gene with either wild-type or mutant C-repeat/DRE sequences in place of the normal UAS (upstream activator sequence) of the *GAL1* promoter (Fig. 1A). Yeast strains carrying these reporter constructs produced low levels of β-galactosidase and formed white colonies on filters containing X-gal. The reporter strains carrying the wild-type C-repeat/DRE sequences were transformed with a DNA expression library that contained random Arabidopsis cDNA inserts fused to the acidic activator domain of the yeast GAL4 transcription factor, "GAL4-ACT" (Fig. 1A). The notion was that some of the clones might contain a cDNA insert encoding a C-repeat/DRE binding domain fused to GLA4-ACT and that such a hybrid protein could potentially bind upstream of the *lacZ* reporter genes carrying the wild type C-repeat/DRE sequence, activate transcription of the *lacZ* gene and result in yeast forming blue colonies on X-gal-treated filters.

Upon screening about 2 x 10^6 yeast transformants, three "positive" cDNA clones were isolated; i.e., the clones caused yeast strains carrying *lacZ* reporters fused to wild-type C-repeat/DRE inserts to form blue colonies on X-gal-treated filters (Fig. 1B). The three cDNA clones did not cause a yeast strain carrying a mutant C-repeat/DRE fused to *LacZ* to turn blue (Fig. 1B). Thus, activation of the reporter genes by the cDNA clones appeared to be dependent on the C-repeat/DRE sequence. Restriction enzyme analysis and

DNA sequencing indicated that the three cDNA clones had an identical 1.8 kb insert (Fig. 2A). One of the clones, designated pACT-11, was chosen for further study.

pACT-11 encodes a 24 kDa polypeptide with an AP2 domain. Our expectation was that the cDA insert in pACT-11 would have a C-repeat/DRE binding domain fused to the yeast GAL4-ACT sequence. However, DNA sequence analysis indicated that an open reading frame of only nine amino acids had been added to the C-terminus of GAL4-ACT. It seemed highly unlikely that such a short amino acid sequence could comprise a DNA binding domain. Also surprising was the fact that about half of the cDNA insert in pACT-11 corresponded to 25s rRNA sequences (Fig. 2A). Further analysis, however, indicated that the insert had an open reading frame, in opposite orientation to the GAL4-ACT sequence, deduced to encode a 24 kDa polypeptide (Fig. 2A-C). The polypeptide has a basic region that could potentially serve as a nuclear localization signal (Raikhel, N., Plant Physiol. **100**:1627-1632 (1992)) and an acidic C-terminal half (pI of 3.6) that could potentially act as an acidic transcription activator domain (Hahn, S., Cell **72**:481-483 (1993)). A Search of the nucleic acid and protein sequence databases indicated that there was no previously described homology of the 24 kDa polypeptide. However, the polypeptide did have an AP2 domain (Jofuku, K. D., et al., The Plant Cell **6**:1211-1225 (1994)) (Fig. 2B, D), a DNA binding motif of about 60 amino acids (Ohme-Takagi, M., et al., The Plant Cell **7**:173-182 (1994)) that is present in numerous plant proteins including the APETALA2 (Jofuku, K. D., et al., The Plant Cell **6**:1211-1225 (1994)), AINTEGUMENTA (Klucher, K. M., et al., The Plant Cell **8**:137-153 (1996); Elliot, R. C., et al., The Plant Cell **8**:155-168 (1996)) and TINY (Wilson, K., et al., The Plant Cell **8**:659-671 (1996)) proteins of Arabidopsis and the EREBPs (ethylene

response element binding proteins) of tobacco (Ohme-Takagi, M., et al., The Plant Cell 7:173-182 (1995)).

The 24 kDa polypeptide binds to the C-repeat/DRE and activates transcription in yeast. We hypothesized that the 24 kDa polypeptide was responsible for activating the *lacZ* reporter genes in yeast. To test this, the *Bcl*I-*Bgl*II fragment of pACT-11 containing the 24 kDa polypeptide, and the *Bgl*II-*Bgl*II fragment containing the 24 kDa polypeptide plus a small portion of the 25s rRNA sequence, was inserted into the yeast expression vector pDB20.1 (Fig. 3). Plasmids containing either insert in the same orientation as the *ADC1* promoter stimulated synthesis of β-galactosidase when transformed into yeast strains carrying the *lacZ* reporter gene fused to a wild-type *COR15a* C-repeat/DRE (Fig. 3). The plasmids did not, however, stimulate synthesis of β-galactosidase when transformed into yeast strains carrying *lacZ* fused to a mutant version of the *COR15a* C-repeat/DRE (Fig. 3). These data indicated that the 24 kDa polypeptide could bind to the wild-type C-repeat/DRE and activate expression for the *lacZ* reporter gene in yeast. Additional experiments indicated that the 24 kDa polypeptide could activate expression of the *lacZ* reporter gene fused to either a wild-type *COR78* C-repeat/DRE (dimer of MT66) or a wild-type *COR15b* C-repeat/DRE (dimer of MT 68) (not shown). A plasmid containing the *Bcl*I-*Bgl*II fragment (which encodes only the 24 kDa polypeptide) cloned in opposite orientation to the *ADC1* promoter did not stimulate synthesis of β-galactosidase in reporter strains carrying the wild-type *COR15a* C-repeat/DRE fused to *lacZ* (Fig. 3). In contrast, a plasmid carrying the *Bgl*II-*Bgl*II fragment (containing the 24 kDa polypeptide plus some 25s rRNA sequences) cloned in opposite orientation to the *ADC1* promoter produced significant levels of β-galactosidase in reporter strains carrying the wild-type *COR15a* C-repeat/DRE (Fig. 3). Thus, a sequence located closely

upstream of the 24 kDa polypeptide was able to serve as a cryptic promoter in yeast, a result that offered an explanation for how the 24 kDa polypeptide was expressed in the original pACT-11 clone.

Gel shift analysis indicates that the 24 kDa polypeptide binds to the C-repeat/DRE. Gel shift experiments were conducted to demonstrate further that the 24 kDa polypeptide bound to the C-repeat/DRE. Specifically, the open reading frame for the 24 kDa polypeptide was inserted into the pET-28a(+) bacterial expression vector (see Materials and Methods) and the resulting 28 kDa fusion protein was expressed at high levels in *E. coli* (Fig. 4). Protein extracts prepared from *E. coli* expressing the recombinant protein produced a gel shift when a wild-type *COR15a* C-repeat/DRE was used as probe (Fig. 5). No shift was detected with BSA or *E. coli* extracts prepared from strains harboring the vector alone, or the vector with an antisense insert for the 24 kDa polypeptide. Oligonucleotides encoding wild-type C-repeat/DRE sequences from *COR15a* or *COR78* competed effectively for binding to the *COR15a* C-repeat/DRE probe, but mutant version of the *COR15a* C-repeat/DRE did not (Fig. 5). These *in vitro* results corroborated the *in vivo* yeast expression studies indicating that the 24 kDa polypeptide binds to the C-repeat/DRE sequence. The 24 kDa polypeptide was thus designated CBF1 (C-repeat/DRE binding factor 1) and the gene encoding it named *CBF1*.

CBF1 is a unique or low copy number gene. The hybridization patterns observed in southern analysis of Arabidopsis DNA using the entire *CBF1* gene as probe were relatively simple indicating that *CBF1* is either a unique or low copy number gene (Fig. 6). The hybridization patterns obtained were not altered if only the 3' end of the gene was used as the probe (the *Eco*RV/*Bgl*II restriction fragment from pACT-11 encoding the acidic region of CBF1, but not the AP2 domain) or if

hybridization was carried out at low stringency (not shown).

CBF1 transcript levels increase slightly in response to low temperature. Northern analysis indicated that the level of *CBF1* transcripts increased about 2 to 3 fold in response to low temperature (Fig. 7B). In contrast, the transcript levels for *COR15a* increased approximately 35 fold in cold-treated plants (Fig. 7C). Only a singly hybridizing band was observed for *CBF1* at either high or low stringency with probes for either the entire *CBF1* coding sequence or the 3' end of the gene (the *Eco*RV/*Bgl*II fragment of pACT-11) (not shown). The size of the *CBF1* transcripts was about 1.0 kb.

DISCUSSION

The C-repeat/DRE is a *cis*-acting regulatory element that stimulates transcription in response to low temperature (Yamaguchi-Shinozaki, K., et al., The Plant Cell **6**:251-264 (1994); and Baker, S. S., et al., Plant Mol. Biol. **24**:701-713 (1994); Jiang, C., et al., Plant Mol. Biol. **30**:679-684 (1996)). In addition, at least certain versions of the element activate transcription in response to dehydration stress and high salinity (Yamaguchi-Shinozaki, K., et al., The Plant Cell **6**:251-264 (1994)). Determining how the C-repeat/DRE stimulates gene expression in response to these environmental factors, and whether cold, dehydration and high salinity affect independent or overlapping regulatory systems, are key issues that need to be addressed. One step toward this end is determining the identity of protein factors that bind to the C-repeat/DRE. Here we described the first such protein, CBF1. We present evidence that CBF1 binds to the C-repeat/DRE both *in vitro* (gel shift assays) and *in vivo* (yeast expression assays). Further, the results demonstrate that CBF1 can activate transcription of reporter genes in yeast that contain the C-repeat/DRE.

It thus seems probable that CBF1 binds to the C-repeat/DRE element in Arabidopsis plants and has a role in regulating transcription. Having available a cDNA clone for CBF1 makes possible a number of strategies to test this hypothesis such as altering the level of CBF1 in transgenic plants (e.g. "antisense" technology) and determining what effects this has on cold- and dehydration-regulated gene expression.

The results of the southern analysis indicate that *CBF1* is a unique or low copy number gene in Arabidopsis. However, the CBF1 protein contains a 60 amino acid motif, the AP2 domain, that is evolutionary conserved in plants (Weigel, D., The plant Cell **7**:388-389 (1995)). It is present in the APETALA2 (Jofuku, K. D., et al., The Plant Cell **6**:1211-1225 (1994)), AINTEGUMENTA (Klucher, K. M., et al., the Plant Cell **8**:137-153 (1996; and Elliot, R. C., et al., The Plant Cell **8**:155-168 (1996)), TINY (Wilson, K., et al., The Plant Cell **8**:659-671 (1996)) and cadmium-induced (Choi, S.-Y., et al., Plant Physiol. **108**:849 (1995)) proteins of Arabidopsis and the EREBPs of tobacco (Ohme-Takagi, M. et al., The Plant Cell **7**:173-182 (1995)). In addition, a search of the GenBank expressed sequence tagged cDNA database indicates that there is one cDNA from *B. napus*, two from *Ricinus communis*, and more than 25 from Arabidopsis and 15 from rice, that are deduced to encode proteins with AP2 domains. The results of Ohme-Takagi and Shinshi (Ohme-Takagi, M., et al., The Plant Cell **7**:173-182 (1995)) indicate that the function of the AP2 domain is DNA-binding; this region of the putative tobacco transcription factor EREBP2 is responsible for its binding to the *cis*-acting ethylene response element referred to as the GCC-repeat. As discussed by Ohme-Takagi and Shinshi (Ohme-Takagi, M., et al., the Plant Cell **7**:173-182 (1995)), the DNA-binding domain of EREBP2 (the AP2 domain) contains no significant amino acid sequence similarities or obvious

structural similarities with other known transcription factors or DNA binding motifs. Thus, the domain appears to be a novel DNA-binding motif that to date, has only been found in plant proteins.

Presumably the binding of CBF1 to the C-repeat/DRE involves the AP2 domain. In this regard, it is germane to note that the tobacco ethylene response element, AGCCGCC, closely resembles the C-repeat/DRE sequences present in the promoters of the Arabidopsis genes *COR15a*, GGCCGAC, and *COR78/RD29A*, TACCGAC. An intriguing possibility thus raised is that CBF1, the EREBPs and perhaps other AP2 domain proteins are members of a superfamily of DNA binding proteins that recognize a family of *cis*-acting regulatory elements having, potentially, CCG as a common core sequence. The notion would be that differences in the sequence surrounding the CCG core element would result in recruitment of different AP2 domain proteins which, in turn, would be integrated into signal transduction pathways activated by different environmental, hormonal and developmental cues. Such a scenario is akin to the situation that exists for the ACGT-family of *cis*-acting elements (Foster et al., FASEB J. **8**:192-200 (1994)). In this case, differences in the sequence surrounding the ACGT core element result in the recruitment of different bZIP transcription factors involved in activating transcription in response to a variety of environmental and developmental signals.

The results of the yeast transformation experiments indicate that CBF1 has a domain that can serve as a transcriptional activator. The most likely candidate for this domain is the acidic C-terminal half of the polypeptide. Indeed, random acidic amino acid peptides from *E. coli* have been shown to substitute for the GAL4 acidic activator domain of GAL4 in yeast (Ma, J. and M. Ptashne, Cell **51**:113-199 (1987)). Moreover, acidic activator domains have been found to function

across kingdoms (Hahn, S., Cell **72**:481-483 (1993)); the yeast GAL4 acidic activator, for instance, can activate transcription in tobacco (Ma, J., et al., Nature **334**:631-633 (1988)). It has also been shown that certain plant transcription factors, such as Vp1 (McCarty, D. R., et al., Cell **66**:895-905 (1991)), have acidic domains that function as transcriptional activators in plants. Significantly, the acidic activation domains of the yeast transcription factors VP16 and GCN4 require the "adaptor" proteins ADA2, ADA3, and GCN5 for full activity (see Guarente, L., Trends Biochem. Sci. **20**:517-521 (1995)). These proteins form a heteromeric complex (Horiuchi, J., et al., Mol. Cell Biol. **15**:1203-1209 (1995)) that bind to the relevant activation domains. The precise mechanism of transcriptional activation is not known, but appears to involve histone acetylation: there is a wealth of evidence showing a positive correlation between histone acetylation and the transcriptional activity of chromatin (Wolffe, A. P., Trends Biochem. Sci. **19**:240-244 (1994)) and recently, the GCN5 protein has been shown to have histone acetyltransferase activity (Brownell, J. E., et al., Cell **84**:843-851 (1996)). Genetic studies indicate that CBF1, like VP16 and GCN4, requires ADA2, ADA3 and GCN5 to function optimally in yeast. The fundamental question thus raised is whether plants have homologs of ADA2, ADA3 and GCN5 and whether these adaptors are required for CBF1 function (and function of other transcription factors with acidic activator regions) in Arabidopsis.

A final point regards regulation of CBF1 activity. The results of the northern analysis indicate that *CBF1* transcript levels increase only slightly in response to low temperature, while those for *COR15a* increase dramatically (Fig. 7). Thus, unlike in yeast, it would appear that transcription of *CBF1* in Arabidopsis at warm temperatures is not sufficient to

cause appreciable activation of promoters containing the C-repeat/DRE. The molecular basis for this apparent low temperature activation of CBF1 in Arabidopsis is not known. One intriguing possibility, however is that CBF1 might be modified at low temperature in Arabidopsis resulting in either stabilization of the protein, translocation of the protein from the cytoplasm to the nucleus, or activation of either the DNA binding domain or activation domain of the protein. Such modification could involve a signal transduction pathway that is activated by low temperature. Indeed, as already discussed, cold-regulated expression of COR genes in Arabidopsis and alfalfa appears to involve a signal transduction pathway that is activated by low temperature-induced calcium flux (Knight, H., et al., The Plant Cell **8**:489-503 (1996); Knight, M. R., et al., Nature **352**:524-526 (1991); Monroy, A. F., et al, Plant Physiol. **102**:1227-1235 (1993); Monroy, A. F., and R. S., The Plant Cell, **7**:321-331 (1995)). It will, therefore, be of interest to determine whether CBF1 is modified at low temperature, perhaps by phosphorylation, and if so, whether this is dependent on calcium-activated signal transduction.

EXAMPLE 2

Use of CBF1 to improve plant stress tolerance

Many plants increase in freezing tolerance in response to low non-freezing temperatures, a process known as cold acclimation. A large number of biochemical changes occur during cold acclimation including the activation of *COR* genes. These genes, which are also expressed in response to dehydration (e.g., drought and high salinity), are thought to help protect plant cells against the potentially deleterious effects of dehydration associated with freezing, drought and high salinity stress. Indeed, expression of the *COR15a* gene in plants grown at normal temperatures (22°C) enhances the freezing tolerance of chloroplasts.

By manipulating the expression of *COR* genes, the stress tolerance of crop and horticultural plants are improved, e.g., engineer broader climate ranges; target stress resistance to stress-sensitive parts of plants; render plants stress-resistant when a stress condition (frost and drought) is imminent. To bring about these effects, however, the expression of the *COR* genes must be manipulated. The gene, *CBF1*, that encodes the transcription factor that binds to the C-repeat/DRE regulatory element present in the promoters of all *COR* genes described to date has been isolated. *CBF1* in yeast activates expression of reporter genes that have been fused to the C-repeat/DRE element. Thus, expression of *CBF1* in plants can activate expression of *COR* genes.

By introducing modified versions of *CBF1* into plants, the expression of *COR* genes can be modified, and thereby enhance the freezing and dehydration tolerance of plants. One potentially useful modification of CBF1 is to place the gene under the control of a strong constitutive promoter. This leads to increased levels of *COR* gene expression in both non-stress and stressed plants which in turn, results in enhanced freezing and dehydration tolerance. Other potential useful modifications are to place *CBF1* under a tissue specific promoter to alter COR gene expression in tissues that are highly sensitive to stress (and thereby enhance the stress tolerance of these tissues); to place CBF1 under control of an inducible promoter such that *COR* gene expression could be induced by application of an exogenous inducer (e.g., induce *COR* genes when a frost is imminent); and modify the activation domain of the CBF1 protein to alter the temperature and dehydration range over which it activates *COR* gene expression. In all of these experiments, modified CBF1 genes are transformed into the plant of interest to modify expression of the endogenous *COR* genes. Of course, it

is also possible to transform into plants various genes fused to the C-repeat/DRE element and control the expression of these genes through the action of *CBF1*.

APPENDIX I

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Michael F. Thomashow and
Eric J. Stockinger

(ii) TITLE OF INVENTION: DNA AND ENCODED PROTEIN WHICH REGULATES COLD AND DEHYDRATION REGULATED GENES

(iii) NUMBER OF SEQUENCES: 11

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Ian C. McLeod
(B) STREET: 2190 Commons Parkway
(C) CITY: Okemos
(D) STATE: Michigan
(E) COUNTRY: USA
(F) ZIP: 48864

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette, 5.25 inch, 360 Kb storage
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: MS-DOS 5.00
(D) SOFTWARE: Wordperfect 5.1

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Ian C. McLeod
(B) REGISTRATION NUMBER: 20,931
(C) REFERENCE/DOCKET NUMBER: MSU 4.1-310

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (517) 347-4100
(B) TELEFAX: (517) 347-4103
(C) TELEX: None

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 905
(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:
(A) ORGANISM: *Arabidopsis thaliana*
(B) STRAIN:
(C) INDIVIDUAL ISOLATE: N/A
(D) DEVELOPMENTAL STAGE: N/A
(E) HAPLOTYPE: N/A
(F) TISSUE TYPE: N/A
(G) CELL TYPE: N/A
(H) CELL LINE: N/A
(I) ORGANELLE: N/A

(vii) IMMEDIATE SOURCE: N/A

(viii) POSITION IN GENOME: N/A

(ix) FEATURE:
(A) NAME/KEY: CBF1 gene
(B) LOCATION:
(C) IDENTIFICATION METHOD: sequencing
(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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TAGTAACTAC GTACTACTTA AACCTTATCC AGTTTCTTGA AACAGAGTAC  150
TCTGATCAAT GAACTCATTT TCAGCTTTTT CTGAAATGTT TGGCTCCGAT  200
TACGAGCCTC AAGGCGGAGA TTATTGTCCG ACGTTGGCCA CGAGTTGTCC  250
GAAGAAACCG GCGGGCCGTA AGAAGTTTCG TGAGACTCGT CACCCAATTT  300
ACAGAGGAGT TCGTCAAAGA AACTCCGGTA AGTGGGTTTC TGAAGTGAGA  350
GAGCCAAACA AGAAAACCAG GATTTGGCTC GGGACTTTCC AAACCGCTGA  400
GATGGCAGCT CGTGCTCACG ACGTCGCTGC ATTAGCCCTC CGTGGCCGAT  450
CAGCATGTCT CAACTTCGCT GACTCGGCTT GGCGGCTACG AATCCCGGAG  500
TCAACATGCG CCAAGGATAT CCAAAAAGCG GCTGCTGAAG CGGCGTTGGC  550
TTTTCAAGAT GAGACGTGTG ATACGACGAC CACGGATCAT GGCCTGGACA  600
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TGGAGGAGAC GATGGTGGAA GCTATTTATA CACCGGAACA GAGCGAAGGT 650

GCGTTTTATA TGGATGAGGA GACAATGTTT GGGATGCCGA CTTTGTTGGA 700

TAATATGGCT GAAGGCATGC TTTTACCGCC GCCGTCTGTT CAATGGAATC 750

ATAATTATGA CGGCGAAGGA GATGGTGACG TGTCGCTTTG GAGTTACTAA 800

TATTCGATAG TCGTTTCCAT TTTTGTACTA TAGTTTGAAA ATATTCTAGT 850

TCCTTTTTTA GAATGGTTCC TTCATTTTAT TTTATTTTAT TGTTGTAGAA 900

ACGAG 905

(3) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 213
(B) TYPE: Amino Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:
(A) ORGANISM: *Arabidopsis thaliana*
(B) STRAIN:
(C) INDIVIDUAL ISOLATE: N/A
(D) DEVELOPMENTAL STAGE: N/A
(E) HAPLOTYPE: N/A
(F) TISSUE TYPE: N/A
(G) CELL TYPE: N/A
(H) CELL LINE: N/A
(I) ORGANELLE: N/A

(vii) IMMEDIATE SOURCE: N/A

(viii) POSITION IN GENOME: N/A

(ix) FEATURE:
(A) NAME/KEY: CBF1 protein
(B) LOCATION:
(C) IDENTIFICATION METHOD: sequencing
(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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15                  20                  25                  30
Lys Pro Ala Gly Arg Lys Lys Phe Arg Glu Thr Arg His Pro Ile Tyr Arg Gly
        35                  40                  45                  50
Val Arg Gln Arg Asn Ser Gly Lys Trp Val Ser Glu Val Arg Glu Pro Asn Lys
                55                  60                  65
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Lys Thr Arg Ile Trp Leu Gly Thr Phe Gln Thr Ala Glu Met Ala Ala Arg Ala
    70                  75                  80                  85
His Asp Val Ala Ala Leu Ala Leu Arg Gly Arg Ser Ala Cys Leu Asn Phe Ala
            90                  95                  100
Asp Ser Ala Trp Arg Leu Arg Ile Pro Glu Ser Thr Cys Ala Lys Asp Ile Gln
105                 110                 115                 120
Lys Ala Ala Ala Glu Ala Ala Leu Ala Phe Gln Asp Glu Thr Cys Asp Thr Thr
        125                 130                 135                 140
Thr Thr Asp His Gly Leu Asp Met Glu Glu Thr Met Val Glu Ala Ile Tyr Thr
                145                 150                 155
Pro Glu Gln Ser Glu Gly Ala Phe Tyr Met Asp Glu Glu Thr Met Phe Gly Met
    160                 165                 170                 175
Pro Thr Leu Leu Asp Asn Met Ala Glu Gly Met Leu Leu Pro Pro Pro Ser Val
            180                 185                 190
Gln Trp Asn His Asn Tyr Asp Gly Glu Gly Asp Gly Asp Val Ser Leu Trp Ser
195                 200                 205                 210
Tyr
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:
(A) ORGANISM: N/A - Synthetic
(B) STRAIN:
(C) INDIVIDUAL ISOLATE: N/A
(D) DEVELOPMENTAL STAGE: N/A
(E) HAPLOTYPE: N/A
(F) TISSUE TYPE: N/A
(G) CELL TYPE: N/A
(H) CELL LINE: N/A
(I) ORGANELLE: N/A

(vii) IMMEDIATE SOURCE: N/A
(viii) POSITION IN GENOME: N/A

(ix) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD: sequencing
(D) OTHER INFORMATION: Table 1

(x) PUBLICATION INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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GATCATTTCA TGGCCGACCT GCTTTTT                         27
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(3) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:
(A) ORGANISM: N/A - Synthetic
(B) STRAIN:
(C) INDIVIDUAL ISOLATE: N/A
(D) DEVELOPMENTAL STAGE: N/A
(E) HAPLOTYPE: N/A
(F) TISSUE TYPE: N/A
(G) CELL TYPE: N/A
(H) CELL LINE: N/A
(I) ORGANELLE: N/A

(vii) IMMEDIATE SOURCE: N/A
(viii) POSITION IN GENOME: N/A

(ix) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD: sequencing
(D) OTHER INFORMATION: Table 1

(x) PUBLICATION INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CACAATTTCA AGAATTCACT GCTTTTTT 28

(4) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:
(A) ORGANISM: N/A - Synthetic
(B) STRAIN:
(C) INDIVIDUAL ISOLATE: N/A
(D) DEVELOPMENTAL STAGE: N/A

(E) HAPLOTYPE: N/A
(F) TISSUE TYPE: N/A
(G) CELL TYPE: N/A
(H) CELL LINE: N/A
(I) ORGANELLE: N/A

(vii) IMMEDIATE SOURCE: N/A
(viii) POSITION IN GENOME: N/A

(ix) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD: sequencing
(D) OTHER INFORMATION: Table 1

(x) PUBLICATION INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GATCATTTCA TGGTATGTCT GCTTTTT 27

(5) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:
(A) ORGANISM: N/A - Synthetic
(B) STRAIN:
(C) INDIVIDUAL ISOLATE: N/A
(D) DEVELOPMENTAL STAGE: N/A
(E) HAPLOTYPE: N/A
(F) TISSUE TYPE: N/A
(G) CELL TYPE: N/A
(H) CELL LINE: N/A
(I) ORGANELLE: N/A

(vii) IMMEDIATE SOURCE: N/A
(viii) POSITION IN GENOME: N/A

(ix) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD: sequencing
(D) OTHER INFORMATION: Table 1

(x) PUBLICATION INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GATCATTTCA TGGAATCACT GCTTTTT 27

(6) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:
(A) ORGANISM: N/A - Synthetic
(B) STRAIN:
(C) INDIVIDUAL ISOLATE: N/A
(D) DEVELOPMENTAL STAGE: N/A
(E) HAPLOTYPE: N/A
(F) TISSUE TYPE: N/A
(G) CELL TYPE: N/A
(H) CELL LINE: N/A
(I) ORGANELLE: N/A

(vii) IMMEDIATE SOURCE: N/A
(viii) POSITION IN GENOME: N/A

(ix) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD: sequencing
(D) OTHER INFORMATION: Table 1

(x) PUBLICATION INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GATCACTTGA TGGCCGACCT CTTTTTT 27

(7) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:
(A) ORGANISM: N/A - Synthetic
(B) STRAIN:
(C) INDIVIDUAL ISOLATE: N/A

(D) DEVELOPMENTAL STAGE: N/A
(E) HAPLOTYPE: N/A
(F) TISSUE TYPE: N/A
(G) CELL TYPE: N/A
(H) CELL LINE: N/A
(I) ORGANELLE: N/A

(vii) IMMEDIATE SOURCE: N/A
(viii) POSITION IN GENOME: N/A

(ix) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD: sequencing
(D) OTHER INFORMATION: Table 1

(x) PUBLICATION INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GATCAATATA CTACCGACAT GAGTTCT 27

(8) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:
(A) ORGANISM: N/A - Synthetic
(B) STRAIN:
(C) INDIVIDUAL ISOLATE: N/A
(D) DEVELOPMENTAL STAGE: N/A
(E) HAPLOTYPE: N/A
(F) TISSUE TYPE: N/A
(G) CELL TYPE: N/A
(H) CELL LINE: N/A
(I) ORGANELLE: N/A

(vii) IMMEDIATE SOURCE: N/A
(viii) POSITION IN GENOME: N/A

(ix) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD: sequencing
(D) OTHER INFORMATION: Table 1

(x) PUBLICATION INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ACTACCGACA TGAGTTCCAA AAAGC 25

(9) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60
(B) TYPE: Amino Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Polypeptide

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:
(A) ORGANISM: *Arabidopsis thaliana*
(B) STRAIN:
(C) INDIVIDUAL ISOLATE: N/A
(D) DEVELOPMENTAL STAGE: N/A
(E) HAPLOTYPE: N/A
(F) TISSUE TYPE: N/A
(G) CELL TYPE: N/A
(H) CELL LINE: N/A
(I) ORGANELLE: N/A

(vii) IMMEDIATE SOURCE: N/A
(viii) POSITION IN GENOME: N/A

(ix) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD: sequencing
(D) OTHER INFORMATION: Figure 2D

(x) PUBLICATION INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Ile Tyr Arg Gly Val Arg Gln Arg Asn Ser Gly Lys
                 5                  10
Trp Val Ser Glu Val Arg Glu Pro Asn Lys Lys Thr
        15                  20
Arg Ile Trp Leu Gly Thr Phe Gln Thr Ala Glu Met
25                  30                  35
Ala Ala Arg Ala His Asp Val Ala Ala Leu Ala Leu
            40                  45
Arg Gly Arg Ser Ala Cys Leu Asn Phe Ala Asp Ser
    50                  55                  60
```

(10) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 61
(B) TYPE: Amino Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Polypeptide

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Tobacco
(B) STRAIN:
(C) INDIVIDUAL ISOLATE: N/A
(D) DEVELOPMENTAL STAGE: N/A
(E) HAPLOTYPE: N/A
(F) TISSUE TYPE: N/A
(G) CELL TYPE: N/A
(H) CELL LINE: N/A
(I) ORGANELLE: N/A

(vii) IMMEDIATE SOURCE: N/A
(viii) POSITION IN GENOME: N/A

(ix) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD: sequencing
(D) OTHER INFORMATION: Figure 2D

(x) PUBLICATION INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
His Tyr Arg Gly Val Arg Gln Arg Pro Trp Gly Lys
                 5                   10
Phe Ala Ala Glu Ile Arg Asp Pro Ala Lys Asn Gly
        15                  20
Ala Arg Val Trp Leu Gly Thr Tyr Glu Thr Ala Glu
25                  30                  35
Glu Ala Ala Leu Ala Tyr Asp Lys Ala Ala Tyr Arg
            40                  45
Met Arg Gly Ser Lys Ala Leu Leu Asn Phe Pro His
    50                  55                  60
Arg
```

WE CLAIM:

-1-

An isolated DNA encoding a protein having a sequence of amino acids essentially homologous to that set forth in SEQ ID NO:2 such that upon providing the DNA encoding the protein in a living plant material a transcription regulating region controlling at least one cold or dehydration regulated gene in the plant material is activated.

-2-

The DNA of Claim 1 wherein the transcription regulating region is identified as C-repeat/DRE of *Arabidopsis thaliana* and essentially homologous regulatory regions in other plant species.

-3-

The DNA of Claim 1 wherein the protein is encoded by 159 to 797 of DNA as set forth in SEQ ID NO:1.

-4-

The DNA of Claim 1 wherein the protein has an amino acid sequence as set forth in SEQ ID NO:2.

-5-

The DNA of Claim 1 wherein the living plant material is *Arabidopsis thaliana*.

-6-

The DNA of Claim 1 wherein the living material is a yeast.

-7-

The DNA of Claim 6 wherein the yeast is *Saccharomyces cerevisiae*.

-8-

An isolated DNA as set forth in 159 to 797 of SEQ ID NO:1.

-9-

A plasmid deposited as ATCC 98063 containing a DNA insert encoding a protein which activates a transcription regulating region identified as the C-repeat/DRE.

-10-

A plasmid containing a DNA insert encoding a protein comprising a sequence of amino acids essentially homologous to that set forth in SEQ ID NO:2 such that upon providing the DNA encoding protein in a living plant material a transcription regulating region controlling at least one cold or dehydration regulated gene in the plant material is activated.

-11-

The plasmid of Claim 10 wherein the DNA insert encodes the protein which activates the transcription regulating region identified as C-repeat/DRE of *Arabidopsis thaliana* and essentially homologous regulatory regions in other plant species.

-12-

The plasmid of Claim 10 wherein the protein is encoded by the DNA which is as set forth in 159 to 797 of SEQ ID NO:1.

-13-

The plasmid of Claim 14 wherein the protein encoded is an amino acid sequence as set forth in SEQ ID NO:2.

-14-

A microorganism containing a plasmid having a DNA insert encoding a protein comprising a sequence of amino acids essentially homologous to that set forth in SEQ ID NO:1 such that upon providing the DNA in a living plant material a transcription regulating region controlling at least one cold or dehydration regulated gene in the living plant material is activated.

-15-

The microorganism of Claim 15 wherein the DNA which encodes the protein which activates the cold regulating region identified as C-repeat/DRE of *Arabidopsis thaliana* and essentially homologous regulatory regions in other plant species.

-16-

The microorganism of Claim 15 wherein the protein is encoded by the DNA which is set forth in 159 to 797 of SEQ ID NO:1.

-17-

The microorganism of Claim 15 wherein the protein encoded by the DNA has an amino acid sequence as set forth in SEQ ID NO:2.

-18-

The microorganism of Claim 15 which is *Escherichia coli*.

-19-

The microorganism of Claim 15 which is *Saccharomyces cerevisiae*.

-20-

A plant material containing a recombinant DNA encoding a protein comprising a sequence of amino acids essentially homologous to that set forth in SEQ ID NO:1 such that a transcription regulating region controlling at least one cold or dehydration regulated gene in the plant material is activated by the protein.

-21-

The plant material of Claim 21 wherein the DNA which encodes the protein which activates the cold regulating region is identified as C-repeat/DRE of *Arabidopsis thaliana* and essentially homologous regulatory regions in other plant species.

-22-

The plant material of Claim 21 wherein the protein is encoded by the DNA which is set forth in 159 to 797 of SEQ ID NO:1.

-23-

The plant material of Claim 21 wherein the protein encoded by the DNA has an amino acid sequence as set forth in SEQ ID NO:2.

-24-

A protein free of other proteins comprising a sequence of amino acids essentially homolgous to that set forth in SEQ ID NO:2 such that upon providing DNA encoding the protein in a living plant material a transcription regulating region controlling at least one cold or dehydration regulated gene in the plant material is activated.

-25-

The protein of Claim 25 wherein the transcription regulating region which is activated is identified as the C-repeat/DRE of *Arabidopsis thaliana* and essentially homologous regulatory regions in other plant species.

-26-

The protein of Claim 25 which is encoded by DNA as set forth in 159 to 797 of SEQ ID NO:1.

-27-

A protein free of other proteins having an amino acid sequence as set forth in SEQ ID NO:2.

-28-

A protein free of other proteins of *Arabidopsis thaliana* having a molecular weight of about 24 kDa as measured in an electrophoresis gel, which binds to a transcription regulating DNA region controlling a cold or dehydration regulated gene of the *Arabidopsis thaliana*.

-29-

A protein as encoded by a DNA in plasmid pEJS251 deposited as ATCC 98063.

-30-

A composition useful for introduction into plants to increase cold or dehydration tolerance of the plant which comprises:

(a) a protein having a sequence of amino acids essentially homologous to that set forth in SEQ ID NO:2 such that upon providing the DNA encoding the protein in a living plant material a transcription regulating region controlling at least one cold or dehydration regulated gene in the plant material is activated; and

(b) a carrier for the introduction of the protein into the plant.

-31-

A composition useful for introduction into plants to increase cold or dehydration tolerance of the plant which comprises:

(a) a protein as encoded by a DNA in a plasmid deposited as ATCC 98063; and

(b) a carrier for the introduction of the protein into the plant.

-32-

A method of regulating cold or dehydration tolerance of a living plant material which comprises:

introducing into the living plant material a DNA encoding a protein comprising a sequence of amino acids essentially homolgous to that set forth in SEQ ID NO:1 such that upon providing the DNA encoding the protein in the living plant material a transcription regulating region controlling at least one cold or dehydration regulated gene in the plant material is activated.

-33-

The method of Claim 33 wherein the DNA which encodes the protein which activates the transcription regulating region is identified as C-repeat/DRE of *Arabidopsis thaliana* and essentially homologous regulatory regions in other plant species.

-34-

The method of Claim 33 wherein the protein is encoded by DNA as set forth in 159 to 797 of SEQ ID NO:1.

-35-

The method of Claim 33 wherein the protein has an amino acid sequence as set forth in SEQ ID NO:2.

-36-

The method of Claim 33 wherein the plant is a crop or horticultural plant.

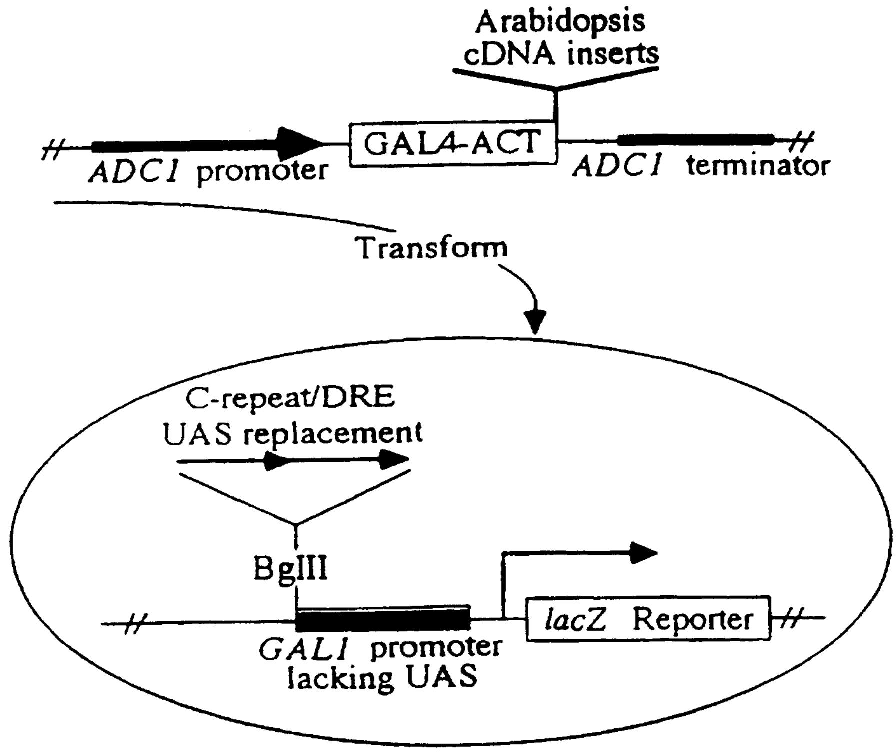

FIG. 1A

Activity of "positive" cDNA clones in reporter strains

UAS Sequences			Yeast colony color on X-gal filters
Oligo	C-repeat/DRE	Inserts	
MT50	*COR15a*		Blue
MT50	*COR15a*		Blue
MT66	*COR78*		Blue
MT52	*M1COR15a*		White

FIG. 1B

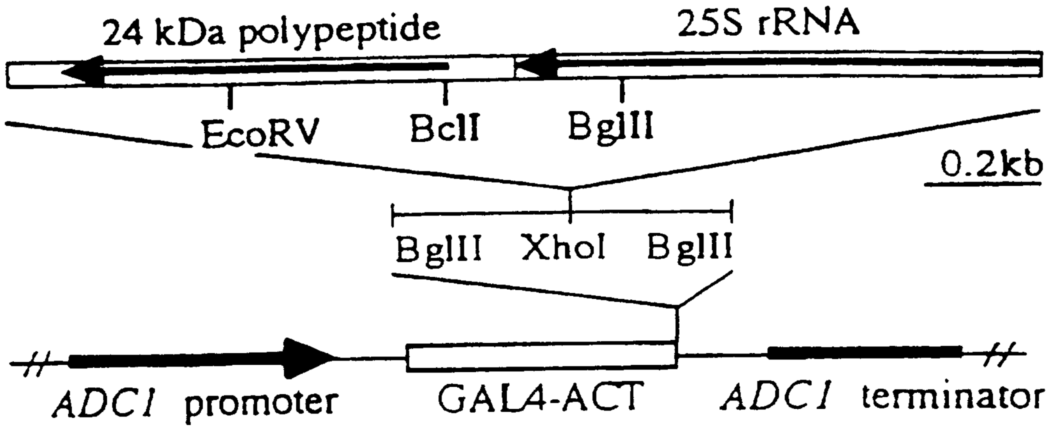

FIG. 2A

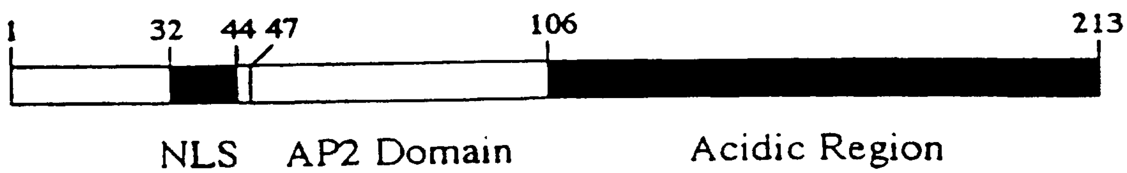

FIG. 2C

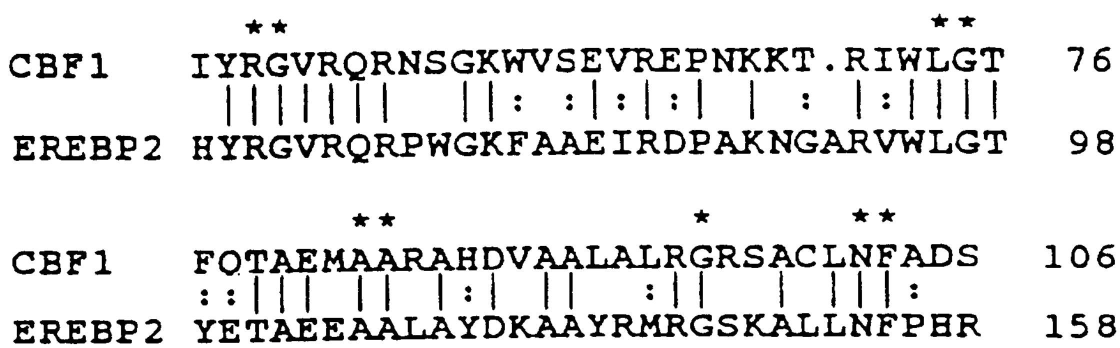

FIG. 2D

```
AAAAAGAATCTACCTGAAAAGAAAAAAAAAGAGAGAGAGATATAAATAGCTTACCAAGACAGATATACTATC   71
TTTTATTAATCCAAAAAGACTGAGAACTCTAGTAACTACGTACTACTTAAACCTTATCCAGTTTCTTGAAA    142
CAGAGTACTCTGATCAATG AAC TCA TTT TCA GCT TTT TCT GAA ATG TTT GGC TCC GAT    200
                 M   N   S   F   S   A   F   S   E   M   F   G   S   D      14

TAC GAG CCT CAA GGC GGA GAT TAT TGT CCG ACG TTG GCC ACG AGT TGT CCG AAG    254
 Y   E   P   Q   G   G   D   Y   C   P   T   L   A   T   S   C   P   K     32

AAA CCG GCG GGC CGT AAG AAG TTT CGT GAG ACT CGT CAC CCA ATT TAC AGA GGA    308
 K   P   A   G   R   K   K   F   R   E   T   R   H   P   I   Y   R   G     50

GTT CGT CAA AGA AAC TCC GGT AAG TGG GTT TCT GAA GTG AGA GAG CCA AAC AAG    362
 V   R   Q   R   N   S   G   K   W   V   S   E   V   R   E   P   N   K     68

AAA ACC AGG ATT TGG CTC GGG ACT TCC CAA ACC GCT GAG ATG GCA GCT CGT GCT    416
 K   T   R   I   W   L   G   T   F   Q   T   A   E   M   A   A   R   A     86

CAC GAC GTC GCT GCA TTA GCC CTC CGT GGC CGA TCA GCA TGT CTC AAC TTC GCT    470
 H   D   V   A   A   L   A   L   R   G   R   S   A   C   L   N   F   A    104

GAC TCG GCT TGG CGG CTA CGA ATC CCG GAG TCA ACA TGC GCC AAG GAT ATC CAA    524
 D   S   A   W   R   L   R   I   P   E   S   T   C   A   K   D   I   Q    122

AAA GCG GCT GCT GAA GCG GCG TTG GCT TTT CAA GAT GAG ACG TGT GAT ACG ACG    578
 K   A   A   A   E   A   A   L   A   F   Q   D   E   T   C   D   T   T    140

ACC ACG GAT CAT GGC CTG GAC ATG GAG GAG ACG ATG GTG GAA GCT ATT TAT ACA    632
 T   T   D   H   G   L   D   M   E   E   T   M   V   E   A   I   Y   T    158

CCG GAA CAG AGC GAA GGT GCG TTT TAT ATG GAT GAG GAG ACA ATG TTT GGG ATG    686
 P   E   Q   S   E   G   A   F   Y   M   D   E   E   T   M   F   G   M    176

CCG ACT TTG TTG GAT AAT ATG GCT GAA GGC ATG CTT TTA CCG CCG CCG TCT GTT    740
 P   T   L   L   D   N   M   A   E   G   M   L   L   P   P   P   S   V    194

CAA TGG AAT CAT AAT TAT GAC GGC GAA GGA GAT GGT GAC GTG TCG CTT TGG AGT    794
 Q   W   N   H   N   Y   D   G   E   G   D   G   D   V   S   L   W   S    212

TAC TAA TATTCGATAGTCGTTTCCATTTTTGTACTATAGTTTGAAAATATTCTAGTTCCTTTTTTAGAA    863
 Y                                                                         213

TGGTTCCTTCATTTTATTTTATTTTATTGTTGTAGAAACGAG                                 905
```

FIG. 2B

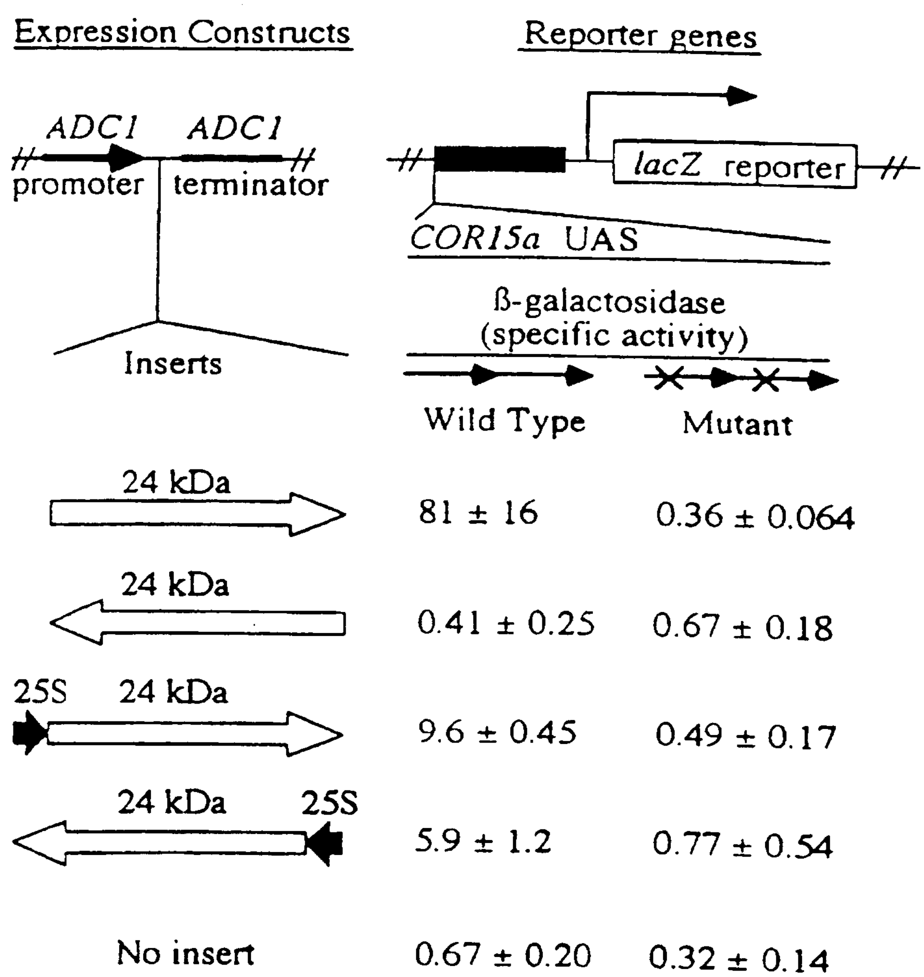
Expression Constructs
Reporter genes
ADC1
ADC1
promoter
terminator
lacZ reporter
COR15a UAS
Inserts
ß-galactosidase
(specific activity)
Wild Type
Mutant
24 kDa
81 ± 16
0.36 ± 0.064
24 kDa
0.41 ± 0.25
0.67 ± 0.18
25S
24 kDa
9.6 ± 0.45
0.49 ± 0.17
24 kDa
25S
5.9 ± 1.2
0.77 ± 0.54
No insert
0.67 ± 0.20
0.32 ± 0.14

FIG. 3

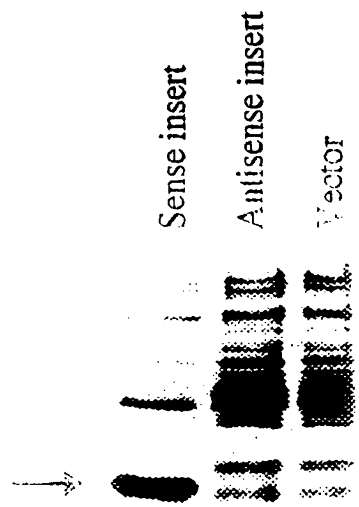

FIG. 4

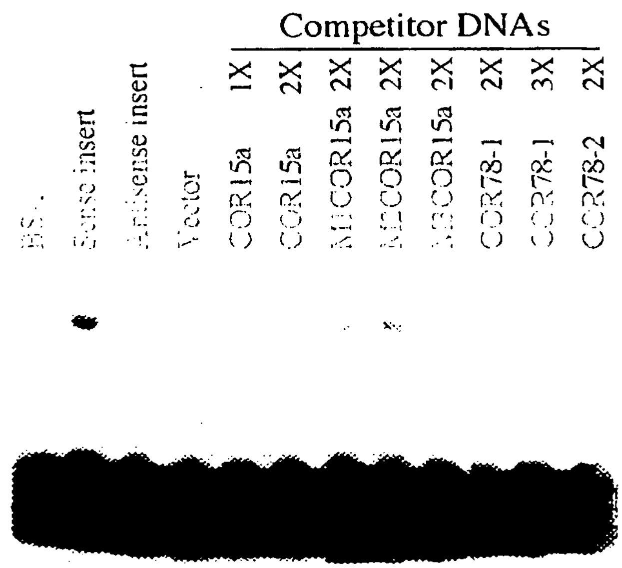
Competitor DNAs
Sense insert
Antisense insert
Vector
COR15a 1X
COR15a 2X
COR78-1 2X
COR78-2 2X

FIG. 5

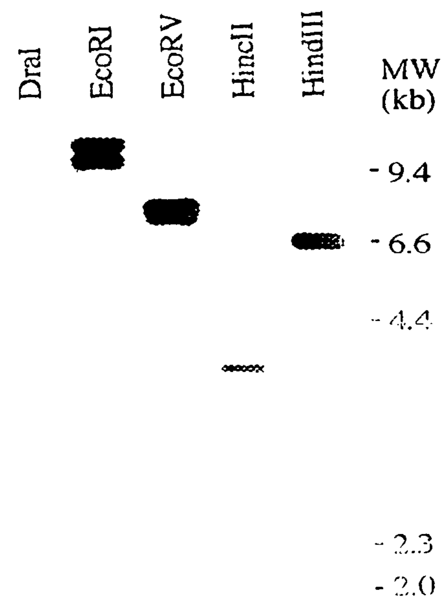

FIG. 6

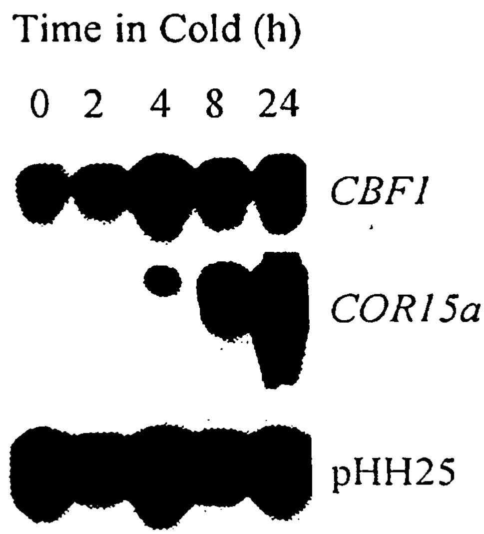

FIG. 7A

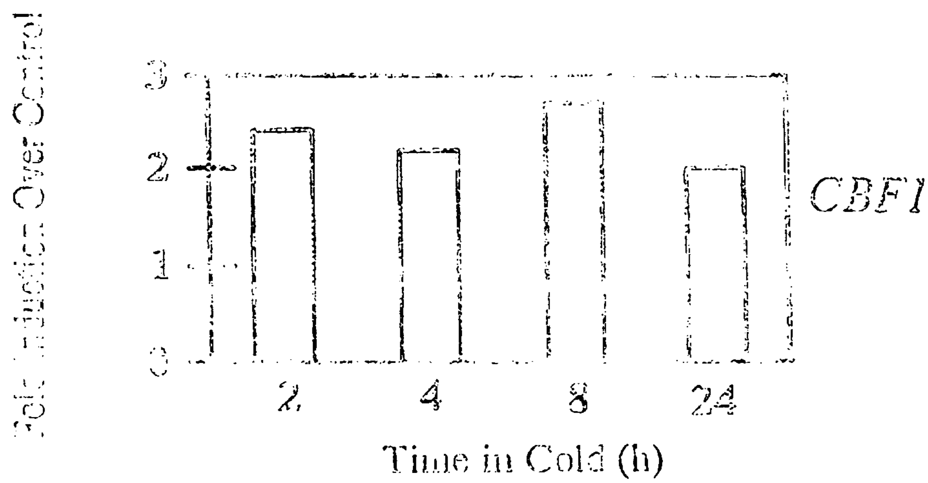

FIG. 7B

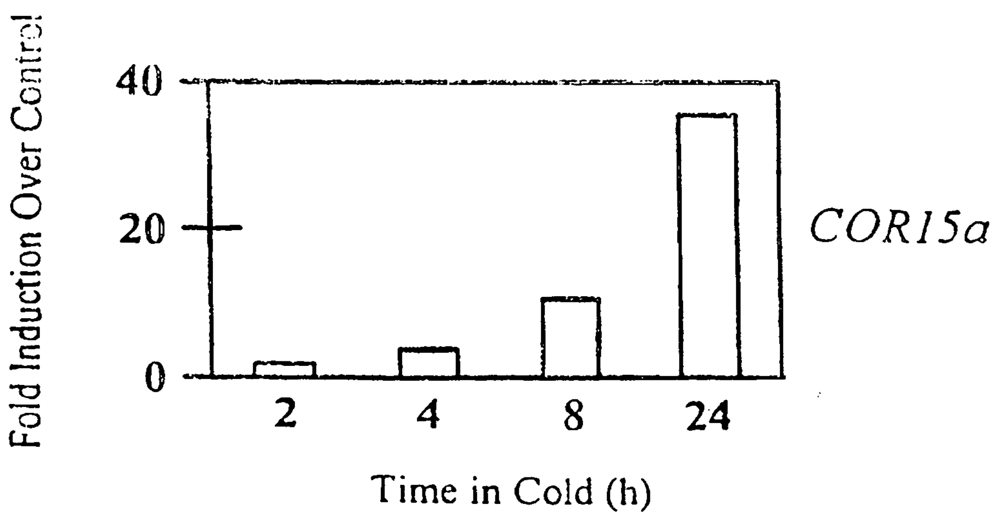

FIG. 7C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/14806

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :514/2, 12; 530/350, 370, 379; 536/23.6
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2, 12; 530/350, 370, 379; 536/23.6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN
search terms: DNA, plant, saccharomyces cerevisiae, yeast, e coli, cold, dehydration

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PERREY et al. Molecular Cloning of a Lupin-Specific Gene From a cDNA Library of Suspension-Cultured Cells of *Lupinus Polyphyllus*. Plant Molecular Biology. 1990. Vol. 15, pages 175-176, especially page 1350.	1 and 4
X	YAMAGUCHI-SHINOZAKI et al. A Novel *cis*-Acting Element in an Arabidopsis Gene Is Involved in Responsiveness to Drought, Low-Temperature, or High-Salt Stress. The Plant Cell. February 1994, Vol. 6, pages 251-264, see entire document.	9

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
06 OCTOBER 1997	30 OCT 1997

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	JEZIA RILEY
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

INTERNAT L SEARCH REPORT

International application No.
PCT/US97/14806

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A01N 37/18; A61K 35/78, 38/00; C07K 1/00; C07H 21/04